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AUTOMATIC DETERMINATION OF SERUM ORNITHINE CARBAMYL TRANSFERASE

STUDY AND TECHNIQUE

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M. L. Girard, F. Rousselet, M. Koch

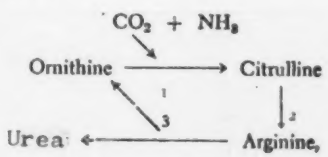
AUTOMATIC DETERMINATION OF SERUM ORNITHINE CARBAMYL TRANSFERASE. STUDY AND TECHNIQUE

Among the substances with which blood serum shows more or less severe hepatic cellular lesions are a certain number of enzymes such as the transaminases, aldosases, dehydrogenases, etc. The measure of enzyme activity thus constitutes an important aspect of the biological exploration of the liver, the value of which is unfortunately limited by the lack of hepatic specificity of the origin of these enzymes and by the difficulty of their determination in the blood. This is with reference to the interest aroused by the work of H. Reichard and P. Reichard [1] on the serum dosage of ornithine carbamyl transferase (OCT), a more specifically hepatic enzyme of ureagenesis found free in the blood in a measurable proportion when there is hepatic cytolysis.

In an attempt to adapt the determination of OCT to automatic analysis it was our purpose to make available to biolgists a simple, and fast, method that could be used to perform a series of analyses and would offer clinicians the possibility of engaging in widespread research and statistical studies.

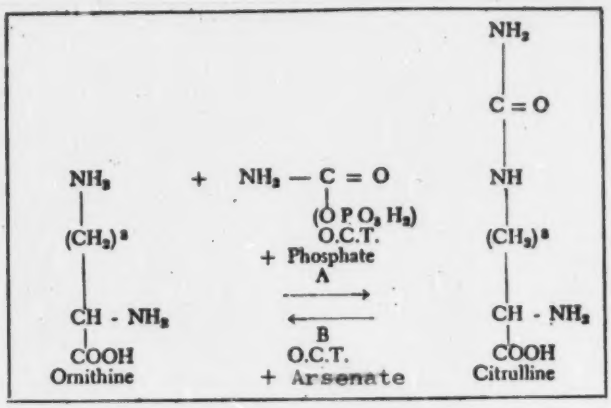
PRINCIPLE

The theoretical studies of Krebs et al [2] on hepatic ureagenesis showed, in the traditional cycle,



the importance of certain amino acids as ammonia vectors, and as enzymes responsible for the direction of the reactions.

Reaction 1, which is more complex than the one shown in the diagram, causes the intervention of carbamyl phosphate, and is seen to be oriented by a transcarbamylase, or OCT.



This reaction is reversible. Krebs [2] has shown that in the presence of an arsenate, reaction B is preponderant. Carbamyl arsenate formed under these conditions is unstable and decomposes spontaneously

$$\text{carbamyl arsenate} \rightarrow CO_2 + NH_3 + \text{arsenate}$$

The reaction oriented in this direction permits either the determination of CO_2 (isotopic method with ^{14}C, hardly applicable as a routine, of H. Reichard and P. Reichard) [1], or the determination of the free ammonia by a sensitive and specific method.

H. Reichard and P. Reichard recommended a microdiffusion method for the ammonia determination, followed by staining by Nessler's reagent [1].

This method was sensitized by G. Demange et al. [3], who substituted Berthelot's phenol hypochlorite reagent for Nessler's reagent.

This technique includes three stages:

the enzyme reaction on a citrulline + arsenate substrate;

ammonia separation by diffusion (Conway cell);

determination of NH_4 + ion by colorimetry.

This method is delicate and the requirement for diffusion in Conway cells makes it difficult to apply in a series of analyses.

This is why, based on earlier work one of us has done [4] we have sought to simplify the technique by recourse to dialysis and to the Technicon autoanalyzer. This method has only two following stages:

enzyme reaction;

direct determination, in the reaction environment, of liberated and dialysed NH_4 +, by the technique of Assous, Dreux and Girard [4] using the Berthelot stain reaction catalysed by sodium nitroprusside.

This method has several advantages, including ease of execution and automation, permitting determinations in long series of analyses, together with satisfactory fidelity and sensitivity.

We thought at first that we would be able to apply this technique directly to the incubation product; serum + citrulline substrate. It has not been possible in fact to operate without changes, because by following the modalities of the enzyme reaction described by Reichard, and those of the technique proposed for the determination on ammoniemia, the color reaction is almost completely inhibited.

That is why we have resumed the study of operating conditions so as to make the determination by automatic dialysis possible, yet retain its biological and clinical meaning.

I. DETERMINATION OF OPERATING CONDITIONS

The impossibility of direct application of dialysis determination of NH_4^+, described above [4], is necessarily related to the presence of one of the two reagents intervening in the composition of the substrate in considerable quantity: citrulline or sodium arsenate.

Experiments have shown that if the arsenate showed a slight inhibiting influence, it was the citrulline in a concentration of 0.2 M in the formula recommended by Reichard that was, in particular, responsible for the inhibition.

In a preceding study [5, 4] one of us indicated the importance of two essential factors in the specific and perceptible determination of ammoniemia by Berthelot's reaction:

the pH, which should be within limits of 9.5 and 10.5;

the chlorometric concentration, which should be between 0.25 and 0.75.

Coloring is not too apparent below the above limits, and the results above these limits are excessively distorted by supplementary ammonia from the reaction between the reagents and the impurities in the environment.

It should be noted however that in this case, the operating conditions are less imperative than in the ammoniemia determination because here we operate comparatively with one indicator which includes the elements of the reaction (blood serum + arsenate), to the exclusion of citrulline.

(1) Preliminary Experiments

We first of all analyzed the phenomenon of inhibition of coloring while

studying, with citrulline present, the modifications of the two important factors in Berthelot's reaction: the pH, and the chlorometric concentration.

(a) Modification of pH

We successively colored 5 µg per ml ammonia solutions at pH 10 and pH 11.5, one in pure water, and the other in a 0.2 M citrulline solution. The optical densities (OD) observed with the Technicon autoanalyzer are listed in Table I.

Table I

Solution	pH	OD	Difference
NH_4^+ 5 µg/ml in H_2O	10	0,140	0,060
	11,5	0,200	
NH_4^+ 5 µg/ml in a 0.5 M citrulline solution	10	0,042	0,038
	11,5	0,080	

Note that the very strong inhibition observed at pH 10 is not diminished by raising the pH to 11.5, and that the increase in the OD is even less for the citrulline solution (0.038) than for distilled water (0.060).

(b) Consumption of Chlorine

The second preliminary experiment actually showed the reality of the consumption of chlorine by the citrulline itself.

The hypochlorite used was a 0.1° chlorometric titrating solution, with a limit value such that the NH_4^+ color can develop.

We obtained the color reaction (Figure 1A) in a trisodium phosphate buffer medium with pH 10.5, first with samples with a range of NH_4^+ comprising 0.25, 0.5, 1, 5, and 10 µg/ml (reference marks, 1, 2, 3, 4, 5), then with two samples of a 0.2 M citrulline solution (reference marks 6, 7). Note that these last points are below the base line; that is, the citrulline consumed the chlorine which had been necessary for the slight coloration due to traces of ammonia always present in the reagents.

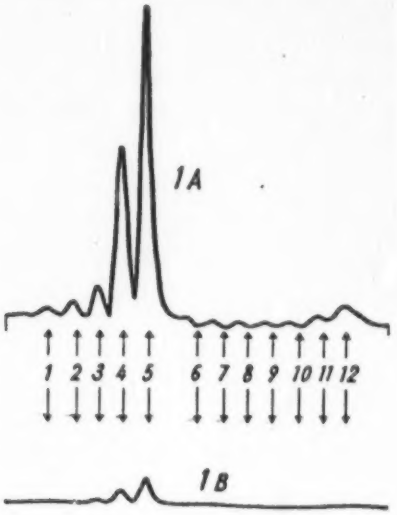


Figure 1. Consumption of chlorine by dialysed citrulline.

A color test in the above range in the presence of citrulline is shown by points 8, 9, 10, 11 and 12. Only the 10 µg/ml sample crossed the base line to any appreciable extent.

By decreasing the pH to 8.30 (disodium phosphate buffer), we obtained, under the same conditions, curve 1B. The color for the range is not appreciable in an aqueous solution expect for the 5 and 10 µg/ml samples. If there is no distortion of the base line in the presence of citrulline, there is no color.

(2) Study of the Influence of the Chlorometric Concentration

We made preceding observations on the one hand to retain a phosphate buffer, the final pH 10 of which is optimum for the coloration of the NH_4^+ ion, and on the other hand to mitigate the consumption of hypochlorite by citrulline by increasing the chlorometric concentration of the reagent used. Such an increase, capable of provoking a slight liberation of the NH_4^+ parasite, seems to be suitable here, contrary to the case of ammoniemia, because of the fact that one sample is used for comparison.

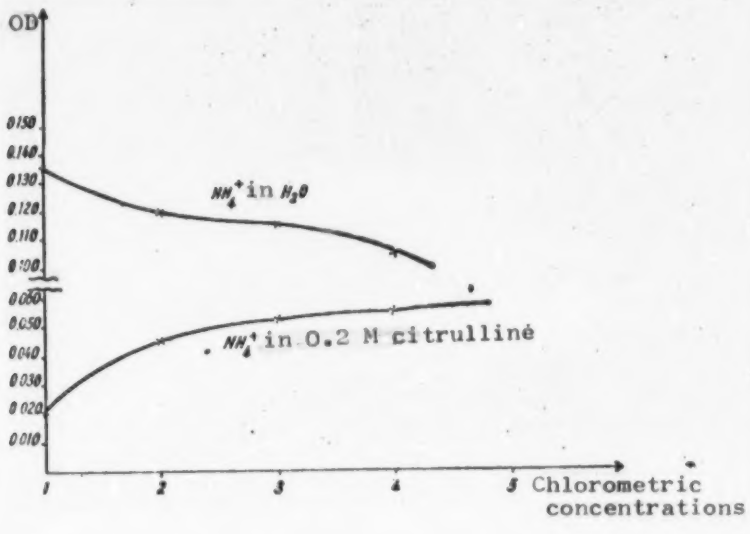


Figure 2. Study of the influence of the chlorometric concentration.

Figure 2 shows the curves for optical densities of the colors obtained with solutions of 5 µg/ml of NH_4^+ in water, or with 0.2 M citrulline added, in terms of the chlorometric concentration.

It thus is evident that the result of using a titrating reagent with a chlorometric concentration of 3 is a clear color for the NH_4^+ ion in the presence of 0.2 M citrulline, without significant disturbance of the

color attributable to NH_4^+ in a pure aqueous solution.

(3) Determination of the Optimum Citrulline Concentration

Experience shows that it is difficult to determine the NH_4^+ in the presence of a high concentration (0.2 M) of citrulline. We had thought that this concentration could be decreased significantly without limiting the enzyme reaction.

It was necessary to use an OCT preparation to verify this hypothesis, and to establish the optimum citrulline concentration.

Lyophylized rabbit liver, an organ rich in OCT, was unsatisfactory because the preparation also included citrullinase, so the quantity of NH_4^+ released was definitely proportional to the citrulline.

We come, finally, to a pool of serums from patients with acute hepatitis in which the very high SGPT transaminases lead us to suppose that the OCT values are equally high.

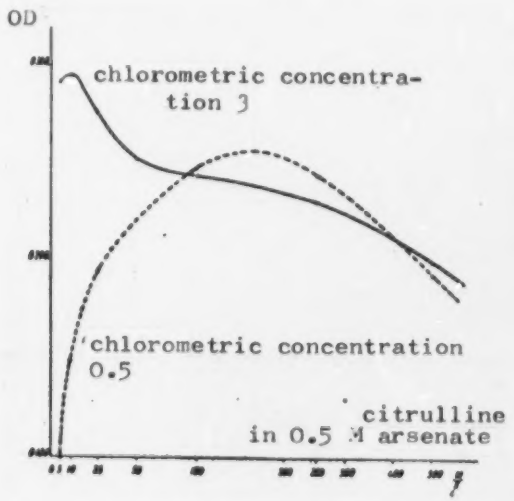


Figure 3. Study of the optimum citrulline concentration.

With different substrates with citrulline concentrations of from M/5 to M/500, the pool of serums used furnished the results shown in Figure 3, where the optical densities are plotted in terms of the reciprocal of the citrulline concentration in 0.5 M arsenate. Each determination was made with a hypochlorite reagent, a titrating phosphate buffer, chlorometric concentration 3 and 0.5, respectively, and providing final pH values of 10.5 and 10 (Figure 3).

The observation shows that the choice of an M/50 citrulline solution as a function of the given substrate has the following advantages:

causes little consumption of chlorine;

provides acceptable color intensity when the titrating reagent used has a chlorometric concentration of 3.

In addition, the concentration adopted is such that enzyme reaction is not limited, nor is its speed influenced. Moreover, simple reasoning, based on the experiment, can justify this latter conclusion. The study of different serums, the very high transaminase levels in which led us to assume significant OCT values, leads us, with the help of substrates with different concentrations of citrulline, to state that the serum OCT would practically never release more than 20 µg of NH_4^+ per ml of serum, which represents about 1 micromole of citrulline. If it is recognized that the molar percentage of transformed citrulline ought not exceed 5%, it would appear that the substrate used should contain 20 micromolesof citrulline per milliliter; that is, a concentration of M/50, to remain in sufficient excess during the enzyme reaction.

These conclusions therefore justify the choice of a titrating hypochlorite reagent with a chlorometric concentration of 3, and a substrate with M/50 citrulline.

These results are also found to be in agreement with the experiments by H. Reichard and P. Reichard concerning the role of the citrulline concentration when the isotopic method is used [1].

(4) Role of the Arsenate Buffer

The authors mentioned above, using the same ^{14}C method, were able to specify the conditions of arsenate concentration for the enzyme reaction dependence.

The arsenate plays a more complex role in the serum OCT determination method by determining liberated ammonia.

(a) Besides the direct action on the enzyme reaction, it is shown to be an inhibitor of secondary ammoniogen reactions (red corpuscles), and thus contributes to the specificity of the determination.

In fact, if one places serum in the presence of an M/50 citrulline solution, or even of an isotonic solution of NaCl, under the same weather conditions, same temperature, and same serum concentration, a higher release of NH_4^+ will often be obtained than what would be produced by the action of serum on the arsenate substrate used.

Here, for example, by way of comparison, are the results, expressed in NH_4^+, obtained in both cases (Table II):

Table II

Number of samples	1	2	3	4	5
Serum + complete substrate	11,5	11,4	3	5	4,4
Serum + citrulline	20,9	12,9	13,5	7,2	7,7

We have retained the 0.5 M concentration for the arsenate proposed by H. Reichard and P. Reichard, because experience has shown that increasing this concentration (to M) causes no change in results, and hence is useless, whereas a decrease in the level is conducive to errors by default.

(b) In addition, the arsenate causes partial inhibition of the coloration attributable to the NH_4^+ ion.

In fact, by effecting in a similar way the reading of a range of NH_4^+ diluted to half with water, and the same range diluted to half by the 0.5 M arsenate buffer, we obtained the results shown in Figure 4. We have, in addition, verified that this inhibiting action of the arsenate on the coloring is found to be definitely independent on the pH, up to a pH of 11.7.

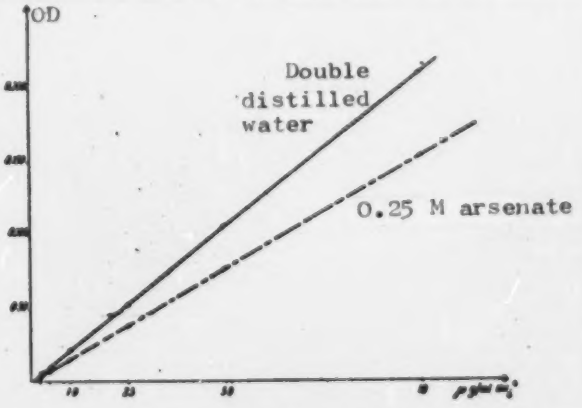


Figure 4. Influence of arsenate on the coloring of ammonia ions by Berthelot's reaction.

The preceding statements led us to use the same concentration of arsenate in the standard solutions as in the determinations.

(5) Influence of the Level of Serum Phosphates

The inhibiting action of phosphates on enzyme reaction has been pointed out by Krebs, Eggleston and Knivett [2], and then by H. Reichard and P. Reichard [1].

Actually, the addition to the reaction medium, citrulline arsenate, of disodium phosphate in a concentration of 0.003 M, that is, at a level three times that of normal serum, will cause no noticeable change in the results.

We can then, in normal practice, disregard the influence of the level of serum phosphates on the reaction.

(6) Study of the Ammoniogen Power of Serum in the Presence of Arsenate Alone

The sample tubes containing arsenate and serum without citrulline showed a significant release of NH_4^+, one corresponding to about 5.20 µg/0.5 ml under operating conditions, that is, after 24 hours in an oven at 37°. This level is deducted from that for the reaction tube to calculate the NH_4^+ actually released under the influence of the OCT.

And we found that in some serums from apparently healthy subjects that were studied as controls, the quantity of NH_4^+ released in the absence of citrulline (control tube) was much higher than that produced in the presence of citrulline, leading to a negative OCT value.

The "inversion" established with the diffusion method, as with the dialysis method, is not an artifact. One is led to think that the citrulline has a slight inhibiting effect on the ammoniogen power of the serum in the presence of arsenate (beyond that of the OCT), or that there is a balance between this power and an NH_4^+ recombination action influenced by an excess of citrulline.

We have sought an inhibiter with this ammoniogen power that would not influence the OCT.

Other than monoiodoacetic acid, the inertness of which we verified, we studied complexon III, seeking to block such metallic ions as Mg^{++}, which could be necessary to this mechanism by analogy with the reaction [6]

$$\text{Ammonia} + \text{biocarbonate} + 2\ \text{ATP} \xrightarrow[+\ Mg^{++}]{\text{acetylglutanate}} \text{carbamylphosphate} + 2\ \text{ADP} + \text{Phosphate}$$

where Mg^{++} is necessary.

As Figure 5 shows, the addition of complexon III to the substrate causes:

a decrease in the quantity of NH_4^+ released in the control tube (serum + arsenate), where the mean is reestablished at from 5.20 µg to 3.45 µg/0.5 ml (comparison 7, 7' and 9, and 11 and 13);

the almost complete disappearance of the inversion phenomena noted above (comparison 10-11 with 12-13).

On the other hand, no decrease in the quantity of NH_4^+ released by the OCT was recorded (comparison of 6, 6', and 8).

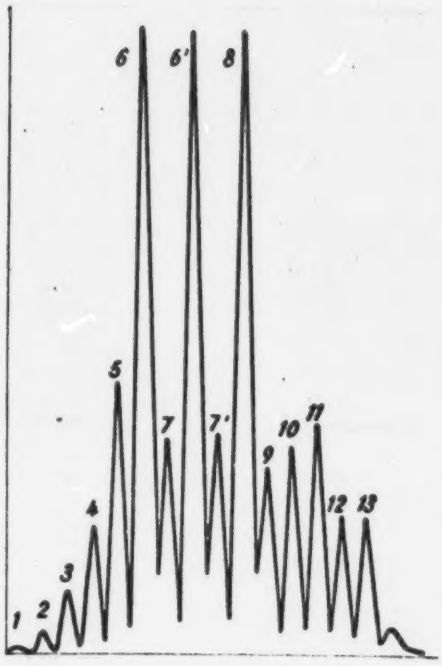


Figure 5. Effect of complexon on the ammoniogen power of the serum in the presence of arsenate.

1, 2, 3, 4, 5 - standardization scale.

6 and 6' - reaction tubes corresponding to one OCT, 2,800 units (without complexon).

7 and 7' - control tubes corresponding to the same serum (without complexon).

8 - same as 6 and 6' with complexon.

9 - same as 7 and 7' with complexon.

10 - reaction tube with a case of "inversion" (without complexon).

A study of this action, and of its mechanism, revealed that the optimum quantity of complexon III to use is of the order of 5 mg per reaction tube.

II. PROPOSED METHOD

(1) Reagents

(A) For enzyme reaction.

R.1 Arsenate buffer 0.5 M, pH 7.15.

Dissolve 15.60 g of crystallized sodium arsenate.

R.P. (Na_2HAsO_4, 7 H_2O) and 0.500 g of complexon III in a quantity sufficient for 100 ml of double distilled water.

Adjust to pH 7.15 with 10 N hydrochloric acid.

R.2 Citrulline substrate M/50.

Dissolve 35 mg of citrulline in 10 ml of arsenate buffer.

(B) For determination of NH_4^+ released.

These are the reagents recommended by Assous, Dreux and Girard [4] except for the phosphate buffer-sodium hypochlorite solution, the titer of which was brought to a chlorometric concentration of 3.

R.3 Sodium chloride solution.

Pure and dry sodium chloride		9 g
Double distilled water	quantity sufficient for	1000 ml

R.4 Sodium phenol solution.

Pure crystallized phenol		25 g
Pure soda in pellets		0.1 g
Double distilled water	quantity sufficient for	1000 ml

pH about 8.

R.5 Sodium nitroprusside solution.

Mother solution: Legal's reagent.

10% aqueous solution of nitroprusside } equal parts
Glacial acetic acid }

Daughter solution: obtained by diluting mother solution to 1/200.

Preservation: one week.

R.6 Phosphate buffer-sodium hypochlorite solution.

Disodium phosphate Na_2HPO_4, $12H_2O$	180 g
Pure soda in pellets	8 g

Double distilled water: about 500 ml

Shake, then add:

Prolabo sodium hypochlorite 10° chlorometric, 300 ml

Bring to 1000 ml with double distilled water and shake until completely dissolved.

R.7 Standard solution of ammonium ions NH_4^+.

Mother solution, 100 µg per ml

Neutral ammonium sulfate, pure and dry	0.3667 g
H_2SO_4 solution, about normal	20 ml
Double distilled water quantity sufficient for	1000 ml

or

Ammonium chloride, pure and dry	0.2972 g
H_2SO_4 solution, about normal	20 ml
Double distilled water quantity sufficient for	1000 ml

Daughter solutions including respectively:

	0.01	0.025	0.05	0.125	0.25	0.50 µ moles NH_4^+/ml
or	0.18	0.45	0.90	2.25	4.50	9 µg of NH_4^+/ml

Measure the corresponding volume of the mother solution into a 100 ml graduate.

Dilute with 40 ml of double distilled water.

Add 50 ml of arsenate buffer.

Bring to 100 ml with double distilled water.

The shelf life of all these solutions is excellent, with exception of the substrate, which can be kept for only a few days in a refrigerator, and the daughter solution of nitroprusside, which must be renewed each week.

(2) Method of Operation

(A) Enzyme reaction.

Measure into a hemolysis tube:

1 ml of serum

1 ml of citrulline substrate M/50 (reagent 2).

Make up at the same time as each determination, a control tube of:

1 ml of serum

1 ml of arsenate buffer (reagent 1).

For each series of determination, prepare two reagent controls:

Substrate control { 1 ml of substrate; 1 ml of double distilled water

Arsenate control { 1 ml of arsenate buffer; 1 ml of double distilled water

All tubes are tightly stoppered, shaken, and kept for 24 hours in an oven at 37°C.

(B) Determination of NH_4^+ Released.

(1) Diagram of Installation (Figure 6).

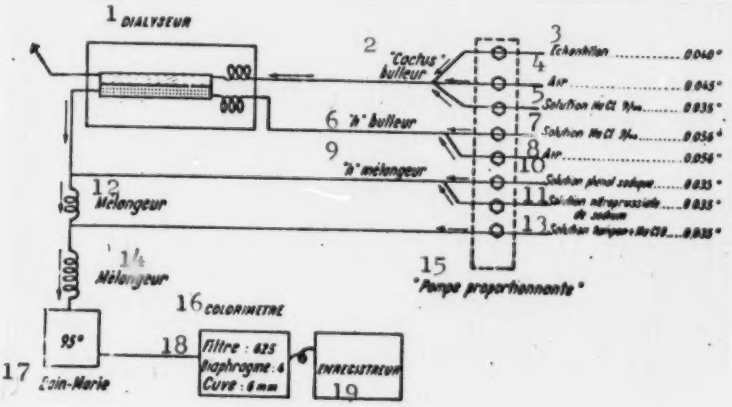


Figure 6. Schematic diagram of the Technicon autoanalyzer.

Key:

1 - dialyser; 2 - "Cactus" bubbler; 3 - sample; 4 - air; 5 - NaCl solution %; 6 - "h" bubbler; 7 - NaCl solution %; 8 - air; 9 - "h" mixer; 10 - sodium phenol solution; 11 - sodium nitroprusside solution; 12 - mixer; 13 - buffer solution + NaClO; 14 - mixer; 15 - "proportioning pump"; 16 - colorimeter; 17 - water bath; 18 - filter: 625 diaphragm: 4 cell: 6 mm; 19 - recorder.

(2) The content of each hemolysis tube after shaking, is poured into a shallow dish on the distributor tray. Interpose a dish of double distilled water between each determination.

(3) Standardization.

A standardization curve is obtained by using the autoanalyzer to read the optical densities obtained for solutions containing 0.01, 0.025, 0.05,

0.0225, 0.25, and 0.50 micromole of NH_4^+/ml.

Likewise, interpose a tube of double distilled water between each tube.

(3) Expression of Results

We have chosen a unit that is easy to use, and that conforms to international conventions, to express serum OCT activity.

The unit is represented by the number of micromoles of citrulline converted at 37° in 24 hours per 1000 ml of serum in the presence of an equal quantity of citrulline substrate M/50-arsenate M/2, buffered to pH 7.15.

The calculation.

Find the OD obtained on the standardized curve by: OD determination - [OD control + (OD citrulline - OD arsenate)].

The number of micromoles read on the curve is multiplied by 2000 to find the method of expression selected.

DISCUSSION

Value of the method.

Linearity:

The results obtained are proportional to the level of serum OCT, as is proven, for example, by the curve obtained by using a pool of normal serums, to dilute a serum from a patient, which is rich in OCT (Figure 7).

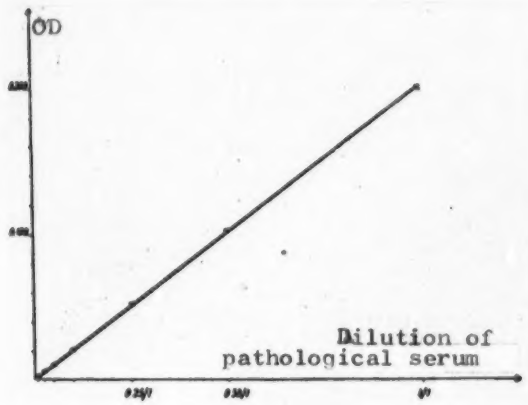


Figure 7. Relation between the quantity of NH_4^+ determined and the enzyme content.

Comparison With the Technique of NH_4^+ Determination by Diffusion:

A series of 20 determinationswas made at the same time by the diffusion method, and by the dialysis method. The results are in agreement within 10 units for all cases, except one, where the deviation was 30 units (50 units found by diffusion, 80 by dialysis).

Normal and Pathological Results

A study of 206 blood donors* showed that 67% had zero serum OCT, and 92% had less than 70 units.

The physiological <u>normality</u> thus was set at 80 units, a value that concords with the conclusions of Demange et al. (one of our units = 7 nanograms [$7 \cdot 10^{-9}$ g] of nitrogen).

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* Through the kindness of Dr. Philippon. Hospital Hemobiology Attache.

A study of over 200 pathological cases* treated for a variety of hepatic ailments, provided a spread of results from 0 to 2200 units, with a maximum of 3000 units in an acute hepatic case.

Comparison of the OCT levels with those of transaminases tends to prove that the OCT determination would provide for greater sensitivity and greater specificity than that of SGPT transaminases during the phenomenon of hepatic lysis.

CONCLUSION

The activity of OCT released into the serum during the course of certain hepatic attacks can be determined in a large series, thanks to the Technicon autoanalyzer.

The method relies on the reversibility of enzyme action in the presence of arsenate (Krebs), and on the photocolorimetric determination of NH_4^+ released, and dialyzed, thanks to Berthelot's phenol-hypochlorite reaction, and in accordance with the modalities of the automatic determination of ammoniemia described by Assous, Dreux, and Girard.

The development of the technique has necessitated study of certain aspects of enzyme reaction, particularly the influence of the constituents of the substrate (citrulline and arsenate) and the ammoniogen power of the serum in the presence of arsenate.

The interference of Complexon III during the secondary reactions has been shown.

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* In collaboration with Prof. Gachin, Hospital Physician (Lariboisiere Hospital).

The possibility of conducting wide-ranging investigations in the field of hepatic pathology has contributed to showing, right from the first series of clinical results, the great sensitivity of the determination in evaluating hepatolysis, thanks to the speed and the simplicity of the method.

SUMMARY

The appearance in blood of ornithine carbamyl transferase seems to be specific evidence of hepactic cytolysis. The methods of estimation of this enzyme proposed by H. Reichard and P. Reichard, and of Ballan, Demange, Moretii et al., based on microdiffusion, have the disadvantages of being relatively time consuming and not applicable to carrying out a large series of cases. A technique of automatic estimation is described using the method of determining ammoniemia by dialysis described by Assous, Dreux and Girard.

The different factors of Berthelot's color reaction have been modified in such a way as to make it possible to carry out the estimation in the presence of excess citrulline introduced by the reagent.

Further, the sensitivity of the technique is increased by the addition of Complexon III, which allows the reduction of the ammoniogenic power of serum in the control reaction (in the absence of citrulline).

Finally, a unit of activity, conforming to international conventions, is proposed.

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